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Expression, regulation, and signaling of the pattern-recognition receptor nucleotide-binding oligomerization domain 2 in rheumatoid arthritis synovial fibroblasts

Ospelt, C ; Brentano, F ; Jüngel, A ; Rengel, Y ; Kolling, C ; Michel, B A ; Gay, R E ; Gay, S

Abstract: **OBJECTIVE:** Since pattern-recognition receptors (PRRs), in particular Toll-like receptors (TLRs), were found to be overexpressed in the synovium of rheumatoid arthritis (RA) patients and to play a role in the production of disease-relevant molecules, we sought to determine the expression, regulation, and function of the PRR nucleotide-binding oligomerization domain 2 (NOD-2) in RA. **METHODS:** Expression of NOD-2 in synovial tissues was analyzed by immunohistochemistry. Expression and induction of NOD-2 in RA synovial fibroblasts (RASFs) were measured by conventional and real-time polymerase chain reaction (PCR) analyses. Levels of interleukin-6 (IL-6) and IL-8 were measured by enzyme-linked immunosorbent assay (ELISA) and expression of matrix metalloproteinases (MMPs) by ELISA and/or real-time PCR. NOD-2 expression was silenced with small interfering RNA. Western blotting with antibodies against phosphorylated and total p38, JNK, and ERK, as well as inhibitors of p38, JNK, and ERK was performed. Activation of NF-kappaB was measured by electrophoretic mobility shift assay. **RESULTS:** NOD-2 was expressed by fibroblasts and macrophages in the synovium of RA patients, predominantly at sites of invasion into articular cartilage. In cultured RASFs, no basal expression of messenger RNA for NOD-2 was detectable, but was induced by poly(I-C), lipopolysaccharide, and tumor necrosis factor alpha. After up-regulation of NOD-2 by TLR ligands, its ligand muramyl dipeptide (MDP) increased the expression of IL-6 and IL-8 via p38 and NF-kappaB. Stimulation with MDP further induced the expression of MMP-1, MMP-3, and MMP-13. **CONCLUSION:** Not only TLRs, but also the PRR NOD-2 is expressed in the synovium of RA patients, and activation of NOD-2 acts synergistically with TLRs in the production of proinflammatory and destructive mediators. Therefore, NOD-2 might contribute to the initiation and perpetuation of chronic, destructive inflammation in RA.

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**Expression, regulation and signaling of the pattern-recognition receptor NOD2 in
rheumatoid arthritis synovial fibroblasts**

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ABSTRACT:

Objective: Since pattern-recognition receptors (PRRs) in particular Toll-like receptors were found to be over-expressed in the synovium of rheumatoid arthritis (RA) patients and to play a role in the production of disease relevant molecules, we analyzed the expression, regulation and function of the PRR NOD2 in RA.

Methods: Expression of NOD2 in synovial tissues was analyzed by immunohistochemistry. Expression and induction of NOD2 in RASFs was measured by conventional and Real-time PCR. Levels of IL-6 and IL-8 were measured by ELISA, expression of MMPs by ELISA and/or Real-time PCR. The expression of NOD2 was silenced with siRNA. Western blots with antibodies against phosphorylated and total p38, JNK and ERK, and inhibitors of p38, JNK and ERK were applied. Activation of NF- κ B was measured by EMSA.

Results: NOD2 was expressed by fibroblasts and macrophages in the synovium of RA patients, predominantly at sites of invasion into articular cartilage. In cultured RASFs no basal expression of mRNA for NOD2 was detectable, but was induced by polyI-C, LPS and TNF. After upregulation of NOD2 by TLR ligands, its ligand MDP increased the expression of IL-6 and IL-8 via p38 and NF- κ B. Stimulation with MDP further induced the expression of MMP1, MMP3 and MMP13.

Conclusion: Not only TLRs but also the PRR NOD2 is expressed in the synovium of RA patients and activation of NOD2 synergistically acts with TLRs in the production of proinflammatory and destructive mediators. Therefore NOD2 might contribute to the initiation and perpetuation of chronic, destructive inflammation in RA.

Activation of the innate immune system is characterized by the detection of pathogens via pattern recognition receptors (PRRs) triggering an inflammatory response. In general, the advancement of this cascade of pro-inflammatory factors leads to clearance of the invading pathogen followed by dissolution of the inflammation. However, growing knowledge of implicated signaling pathways give evidence for more intricate mechanisms involved in physiological as well as pathological activation of innate immunity. The discovery of endogenous ligands for most of the PRRs lead to the hypothesis that in addition to exogenous pathogens, PRRs might response to damaged tissue and parts of apoptotic-cell debris. Furthermore, activation of PRRs has been found to modulate adaptive immune responses through the regulation of co-stimulatory molecules, maturation of dendritic cells and stimulation of B-cells (1, 2). Thus, it is feasible to assume that activation of innate immune receptors has a role in triggering autoimmune diseases such as rheumatoid arthritis (RA).

The family of PRRs comprises membrane-bound receptors such as the well-characterized Toll-like receptors (TLRs) and cytoplasmic receptors like the more recently discovered group of nucleotide-binding domain and LRR receptors (NLRs). NLRs have similarities to TLRs in regard to their leucine-rich-repeat (LRR) domain mediating ligand binding. Their ligands, structure and signaling pathways however differ. There are two major subfamilies of NLRs, NODs and NALPs. NOD1 recognizes products from Gram-negative bacteria (diaminopimelic acids), while NOD2 senses murayml dipeptide (MDP), a peptidoglycan derived peptide from Gram-negative as well as Gram-positive bacteria. Up to now, NODs have mainly been described on antigen-presenting cells and epithelial cells of the intestines (3-5). After binding of their ligands, NOD receptors signal by recruiting the serine threonine kinase RICK, also called RIP2, which then in turn activates TAK1. Further downstream, NOD signaling was shown to lead to activation of NF κ B and mitogen activated kinases (MAPKs) (6).

The active role of synovial fibroblasts in inflammation and joint destruction during the course of RA has been documented in a variety of studies (7). Synovial fibroblasts from RA patients (RASFs) express functional TLR2, TLR3 and TLR4, the activation of which leads to secretion of pro-inflammatory cytokines, chemokines and matrix degrading enzymes (8-10). Therefore, RASFs emerge as cells of the innate immune system which might take part in the initiation and perpetuation of the chronic, destructive inflammation in RA.

In the current study, we show the expression of the PRR NOD2 in the synovium of RA patients and explore its induction, signaling pathways and functionality in RASFs.

MATERIAL AND METHODS

Patients and tissue preparation: Synovial tissues from patients with RA and osteoarthritis (OA) were collected after written consent during joint replacement surgery (Schulthess Clinic Zurich, Switzerland). All RA patients fulfilled the American College of Rheumatology criteria for the classification of RA. For immunohistochemistry, synovial tissues were fixed in formalin and embedded in paraffin. For cell culture, synovial fibroblasts were isolated and cultured as described before (11). Cultured synovial fibroblasts were used for experiments after 4 to 9 passages.

Immunohistochemistry: After deparaffinization, sections were washed in phosphate buffered saline (PBS) and treated with 1% H₂O₂ to disrupt endogenous peroxidase activity. Unspecific protein binding was blocked with 1% bovine serum albumin (BSA)/5% goat serum for one hour. Polyclonal rabbit anti-NOD2 antibodies (Abcam, Cambridge, UK) or normal rabbit serum were applied (1:4000 in 1% BSA) for 1h at room temperature. Slides were washed in PBS/0.05% Tween® and incubated with biotinylated goat anti-rabbit antibodies (Jackson ImmunoResearch, Suffolk, UK) for 30 min. The signal was amplified with horseradish-peroxidase (HRP)-conjugated streptavidin (Vectastain Elite ABC kit, Vector laboratories, Burlingame, CA). Sections were developed with aminoethylcarbazole

chromogen and counterstained with hematoxylin. To show specificity of the rabbit anti-human antibodies, pre-incubation of the antibodies with a synthetic peptide, corresponding to 14 amino acids of NOD2 (Abcam, Cambridge, UK) was performed (30 min, 37°) and staining procedures were continued as described above. Simultaneous NOD2/CD68 and NOD2/vimentin double staining was performed using the NOD2 staining protocol as described above together with mouse anti-CD68 or mouse anti-vimentin primary antibodies. Alkaline-phosphatase (AP) conjugated goat anti-mouse antibodies were applied (Jackson ImmunoResearch, Suffolk, UK). BCIP/NBT was used as substrate for AP, NovaRed as substrate for HRP. Slides were counterstained with methyl green (all Vector laboratories, Burlingame, CA)

Stimulation experiments: Synovial fibroblasts were stimulated with the following reagents: 10 ng/ml TNF α , 1 ng/ml IL-1 β (both R&D Systems, Minneapolis, MN), 300 ng/ml bacterial lipopeptide (bLP) palmitoyl-3-cysteine-serine-lysine-4, 10 μ g/ml polyI-C (PIC) (both InvivoGen, San Diego, CA), 100 ng/ml lipopolysaccharide (LPS) from *Escherichia coli* (List Biological Laboratories, Campbell, CA), 100U/ml IFN β (R&D Systems, Minneapolis, MN) and/or 10 μ g/ml muramyl dipeptide (MDP) N-Acetylmuramyl-L-alanyl-D-isoglutamine hydrate (Sigma, Basel, Switzerland). SB203580 (Calbiochem, San Diego, CA) and SP600125 (Merck, Darmstadt, Germany) were used at 10 μ M, PD98059 (Calbiochem, San Diego, CA) at 25 μ M.

Conventional PCR: Total RNA was isolated using the RNeasy MiniPrep kit (Qiagen, Basel, Switzerland) including DNase treatment. RNA was reverse transcribed and NOD2 transcripts were amplified using primers spanning a 317bp fragment (forward primer: GAATGTTGGGCACCTCAAGT; reverse primer: CAAGGAGCTTAGCCATGGAG). Reaction products were separated on a 1% agarose gel and signals were visualized using ethidium bromide.

Enzyme linked immunosorbant assay (ELISA): Levels of IL-6 and IL-8 in cell culture supernatants were quantified using OptEIA® kits (BD Pharmingen, San Diego, CA). Levels of MMP3 were determined with a DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

siRNA: Commercially available siRNA against NOD2 (Santa Cruz Biotechnology, Santa Cruz, CA) or scrambled RNA control reagent (Ambion, Austin, TX) were transfected by nucleofection at a concentration of 50pmols/5 x 10⁵ cells using the Basic Nucleofector® Kit for primary mammalian fibroblasts (Amaxa, Cologne, Germany).

Electrophoretic Mobility-Shift Assay (EMSA): Nuclear extracts were prepared using a nuclear/cytosol fractionation kit (Biovision, Mountain View, CA). The binding of NF-κB to DNA after MDP stimulation was visualized with an Electrophoretic Mobility Shift Assay (Panomics, Redwood City, CA). Specificity of the complex was confirmed by adding cold probe and 1 μg mouse anti-human NFκB p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) respectively.

Western blot: Cells were lysed in 2x Laemmli buffer, and proteins were denaturated by incubation at 95°C for 3min. Samples were applied to SDS-PAGE and elektroblotted onto Protran nitrocellulose transfer membranes (Schleicher & Schüll, Dassel, Germany). After blocking with 5% non-fat dry milk, membranes were incubated with rabbit anti-human antibodies directed against NOD2 (Santa Cruz Biotechnology, Santa Cruz, CA), or against phosphorylated sites of p38, JNK or ERK (all Cell Signaling Technology, Danvers, MA). As secondary reagents HRP-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) were used, and signals were visualized by enhanced chemoluminescence system (Amersham Biosciences, Otelfingen, Switzerland). For normalization, membranes were stripped and probed with mouse anti-human α-tubulin (Sigma, Basel, Switzerland) or rabbit anti-human p38, ERK or JNK antibodies (Cell Signaling Technology, Danvers, MA). The intensity of the signal was measured by densitometric measurements.

Real-time PCR: Total RNA was isolated using the RNeasy MiniPrep kit (Qiagen, Basel, Switzerland) including DNase treatment, reverse transcribed and used for relative quantification of mRNA levels by Taqman/SYBR green Real-time PCR using eukaryotic 18S rRNA as endogenous control (Applied Biosystems, Rotkreuz, Switzerland).

NOD2: forward TTC TCC GGG TTG TGA AAT GT; reverse CTC CTC TGT GCC TGA AAA GC; **MMP1:** forward TGT GGA CCA TGC CAT TGA GA; reverse TCT GCT TGA CCC TCA GAG ACC; probe AGC CTT CCA ACT CTG GAG TAA TGT CAC ACC; **MMP3:** forward GGG CCA TCA GAG GAA ATG AG; reverse CAC GGT TGG AGG GAA ACC TA; probe AGC TGG ATA CCC AAG AGG CAT CCA CAC; **MMP9:** forward GGC CAC TAC TGT GCC TTT GAG; reverse GAT GGC GTC GAA GAT GTT CAC; probe TTG CAG GCA TCG TCC ACC GG; **MMP13:** forward TCC TAC AAA TCT CGC GGG AAT; reverse GCA TTT CTC GGA GCC TCT CA; probe CAT GGA GCT TGC TGC ATT CTC CTT CAG; **MMP14:** forward TGG AGG AGA CAC CCA CTT TGA ; reverse GCC ACC AGG AAG ATG TCA TTT C ; probe CCT GAC AGT CCA AGG CTC GGC AGA.

Statistical analysis: Values are presented as mean \pm SEM. Wilcoxon signed rank test or Mann-Whitney U test was applied to analyze results for significant differences ($p < 0.05$) using GraphPad Prism Software.

RESULTS

Expression of NOD2 in the synovium of RA and OA patients. Immunohistochemical staining of synovial tissues showed clear expression of NOD2 in 9 out of 11 RA patients, but only in 1 out of 7 OA patients (Figure 1A-D). NOD2 in RA synovial tissues was mainly localized at sites of cartilage and bone destruction, sporadically NOD2 was also found in the lining and sublining layers. Lymphocytic infiltrates or blood vessels did not stain with anti-NOD2 antibodies. Double stainings with NOD2 and CD68 or vimentin,

showed that NOD2 is expressed by both, macrophages as well as by fibroblasts (Figure 2A,B).

Expression and induction of NOD2 in synovial fibroblasts. To analyse whether NOD2 is produced by fibroblasts *in vitro* and how its expression can be induced, we stimulated cultured synovial fibroblasts with the pro-inflammatory cytokines TNF α and IL-1, and with the TLR2 ligand bLP, the TLR3 ligand PIC and the TLR4 ligand LPS. Levels of TNF α and IL-1 are high in patients with RA and these two cytokines have been shown to induce expression of a variety of pro-inflammatory mediators in RA (12). TLR2, TLR3 and TLR4 are the most abundantly expressed TLRs on RASF. Stimulation with the respective ligands was shown to induce the expression of chemokines, cytokines and matrix degrading enzymes (10, 13). After conducting conventional PCR, we found that synovial fibroblasts didn't express mRNA for NOD2 constitutively. However, its transcription could be induced by all used stimuli (Figure 3A). To see differences in the induction of NOD2 mRNA between RASFs and OASFs, mRNA levels after stimulation were quantified by Real-time PCR. Hereby, a stronger induction of NOD2 transcription in RASFs compared to OASFs was visible (Figure 3B). In the analyzed cultures, only after stimulation with PIC this difference between RASF and OASF was constant and therefore statistically significant. Since PIC and LPS both result in activation of the TRIF pathway and production of type I interferons (IFN), we measured whether maybe IFN β might be responsible for the induction of NOD2. However, mRNA levels of NOD2 were not induced after stimulation of RASF with 100U/ml IFN β (data not shown). Increased levels of NOD2 were also seen on protein level by incubation of RASFs with TNF, IL-1, bLP, PIC or LPS for 48h (Figure 3C).

Production of cytokines after stimulation with MDP. To test the functionality of NOD2 receptors on synovial fibroblasts, RASFs were stimulated with TNF α , IL-1, bLP, PIC or LPS alone or in combination with the NOD2 ligand MDP for 24h. Levels of IL-6 and IL-8 were measured in the supernatants by ELISA. Since no constitutive expression of the receptor

NOD2 has been measured on RASFs, MDP alone had no effect on the production of IL-6 or IL-8, as expected. When MDP was added in combination with the stimuli that had induced the expression of NOD2, namely TNF α , IL-1, bLP, PIC and LPS, RASFs produced higher amounts of IL-6 and IL-8 than with the mentioned stimuli alone (Figure 4A, B). The increase in IL-6 and IL-8 production after addition of MDP was significant for all tested stimuli, except for IL-6 levels after stimulation with IL-1 and MDP. The same experiments done with OASFs lead to similar results (data not shown).

In the next experiment, RASFs were stimulated with PIC for 5h to induce the expression of NOD2, washed with PBS and then incubated with increasing concentrations of MDP for 24h. Measurements of IL-6 and IL-8 in the supernatants indicated a dose-dependent and significant increase in the levels of these cytokines by stimulation with MDP (Figure 4C).

Further confirmation that NOD2 signaling regulates the production of IL-6 and IL-8 was obtained by specific downregulation of NOD2 by siRNA. 48h after transfection of RASFs with NOD2 targeted siRNA or scrambled siRNA, using the same stimulation scheme as mentioned above (5h PIC pre-stimulation, 24h MDP stimulation), IL-6 levels were reduced by 22% (48.3 ± 15.9 pg/ml vs 37.5 ± 15 pg/ml) and IL-8 levels by 25% (67.6 ± 26.1 pg/ml vs 50.9 ± 22.8 pg/ml) (Figure 4D). The mean expression of NOD2 mRNA in silenced cells was 60% less than in cells transfected with control RNA as measured by Real-time PCR.

NOD2 signaling pathways. To elucidate the signaling pathways that are activated by the binding of MDP to NOD2, we first analyzed members of the mitogen-activated protein kinase (MAPK) pathway by performing Western blots with protein samples from RASFs that were stimulated for 5h with PIC followed by 15, 30, or 60 min stimulation with MDP. Whereas the ratio between phosphorylated/total protein only slightly increased in the case of c-jun N-terminal kinase (JNK) and extracellular-signal regulated kinase (ERK), phosphorylation of p38 after MDP stimulation was clearly visible (Figure 5A).

In accordance with the results from the Western blots, addition of the p38 inhibitor SB203580 to 24h MDP stimulation (5h PIC pre-stimulation) almost completely blocked IL-6 and IL-8 production, whereas the ERK inhibitor PD98059 or the JNK inhibitor SP600125 had little or no effect (Figure 5B).

In addition to p38 MAPK activation, MDP stimulation after 5h PIC pre-stimulation lead to increased binding of NF- κ B to its DNA binding sites as shown by EMSA (Figure 5C).

Production of matrix-metalloproteinases (MMPs) after stimulation with MDP.

Since RASFs also contribute to pathological matrix degradation by secretion of MMPs, we analyzed whether stimulation with MDP modulates the expression of MMPs. Levels of MMP1, MMP3, MMP9, MMP13 and MMP14 mRNA were measured in RASFs stimulated with PIC alone or in combination with MDP. Levels of MMP9 and MMP14 mRNA did not change after addition of MDP, while levels of MMP1, MMP3 and MMP13 were significantly increased when MDP was added (Figure 6A). Stimulation of MMP3 secretion by MDP was verified on the protein level with ELISA (Figure 6B). Furthermore, silencing of NOD2 by siRNA lead to significant reduction of mRNA expression for MMP1, MMP3 and MMP13 after pre-stimulation with PIC for 5h and subsequent 24h stimulation with MDP in RASFs (Figure 6C).

DISCUSSION:

In the present work, we show elevated expression of the pattern-recognition receptor NOD2 in the synovium of RA patients. Furthermore, we provide evidence that NOD2 activation in synovial fibroblasts leads to the expression of pro-inflammatory cytokines and matrix-degrading enzymes via MAPK and NF- κ B signaling pathways.

Up to now expression of NOD2 has mainly been analyzed in the context of Crohn's disease, since a polymorphisms in the gene encoding NOD2, CARD15, was found to be correlated with higher susceptibility to develop this inflammatory bowel disease in

Caucasians (14). In this respect NOD2 expression has been shown in intestinal mononuclear and endothelial cells and in Paneth cells (4, 15, 16). Furthermore, expression of NOD2 was found in oral epithelial cells and fibroblasts, osteoblasts and trophoblast cells (17-20). These studies, including our work point to the fact that resident tissue cells are able to express innate immune receptors and take an active role in the recognition and response to invading pathogens. The high expression of NOD2 in RA, along with previously shown increased expression of TLR2, TLR3 and TLR4 underscores the importance of activation of innate immune mechanisms in RA (21). However, endogenous ligands such as RNA from necrotic cells for TLR3 or fibrin for TLR4, have yet to be found for NOD2 (10, 22). Nevertheless it is feasible to assume that in the pathogenesis of RA an initial infection or certain bacterial/viral components lead to activation of innate immune receptors which provokes an inflammatory reaction. Due to the inflammation, endogenous ligands, so called DAMPs (danger associated molecular pattern molecules) get released from the tissue and further stimulate innate immune reactions in an positive feedback mechanism. In this respect, our data clearly shows that activation of TLRs leads to increased expression of NOD2. Thereby the repertoire of immune receptors in the cell is widened, making it more sensitive to react to invading pathogens and/or endogenous danger signals.

A central role of synovial fibroblasts in this inflammatory activation is suggested by their production of a wide panel of chemokines, pro-inflammatory cytokines and matrix-degrading enzymes after stimulation of PRRs as shown in the current study and in others (9, 10). This finding assigns different functions to synovial fibroblasts than to epithelial cells, where no production of pro-inflammatory cytokines after stimulation of PRRs was found (23). Most interestingly, cultured synovial fibroblasts from RA patients appear to respond even stronger to activation compared to synovial fibroblasts from OA patients, as we show here for the expression of NOD2. This phenomenon was previously shown for a variety of molecules

and was ascribed to an intrinsic, inflammation-independent activated phenotype of these cells (7).

The induction of pro-inflammatory cytokines by NOD2 signaling has been shown before and a synergistic effect of NODs and TLRs has been suggested (24-26). Since TLR activation readily induced NOD expression and, after induction of NOD2, MDP alone could induce IL-6 and IL-8, we think that the synergistic effect in synovial fibroblasts is mainly based on the up-regulation of NOD2 receptor by TLR ligands. On the other hand, MDP stimulation had no influence on the expression of TLR2, TLR3 or TLR4 on synovial fibroblasts (data not shown).

Regarding NOD2 signaling pathways, we could show that NOD2 stimulation in synovial fibroblasts leads to the activation of NF- κ B as well as of MAPK, in particular p38. NF- κ B activation by MDP has been previously shown and failure of activation of this pathway due to NOD2 mutations has been linked to the pathogenesis of Crohn's disease (27). The activation of MAPK via NOD2 has been shown in murine macrophages, where p38, ERK and JNK were phosphorylated after stimulation with MDP (6, 28). In synovial fibroblasts we mainly found p38 MAPK to be activated by MDP/NOD2. However, indirect mechanisms for the observed increase in MAPK phosphorylation can not completely be ruled out by this experiment. Regarding the strong inhibition of IL-6/IL-8 production by SB203580, it has to be taken into account that SB203580 has been shown not only to inhibit p38 but also the signaling molecule RICK, also called Rip2. Since RICK has a key role in transferring signals after engagement of MDP to NOD2, its inhibition surely has an influence on cytokine production after MDP stimulation (29).

Taken together, our study gives evidence that in addition to TLRs, other PRRs are expressed in RA and that their activation on synovial fibroblasts, possibly by yet unknown endogenous ligands, might lead to perpetuation of inflammation and matrix destruction.

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FIGURE LEGENDS

Figure 1. Expression of NOD2 in RA and OA synovial tissues. Representative pictures of immunohistochemical staining of NOD2 in **A)** RA (n=11) and **C)** OA (n=7) synovial tissues. **B)** RA tissue isotype control, insert showing lack of staining after incubation with a NOD2 blocking peptide; **D)** OA tissue isotype control. Pre-incubation of the antibodies with a synthetic NOD2 peptide blocked staining by rabbit anti-human NOD2 antibodies as shown in inserts of A and B. Magnification 100x, positive signals appear in red, nuclei were counterstained with hematoxylin.

Figure 2. NOD2 is expressed in fibroblasts and macrophages in the synovium. Representative pictures of immunohistochemical double staining of RA synovial tissues (n=4) with NOD2 (red signal) and **A)** CD68 (blue signal), and **B)** vimentin (blue signal). Magnification 100x, magnification in inserts 630x. Nuclei were counterstained with methyl green.

Figure 3. Expression of NOD2 in RASFs is up-regulated by pro-inflammatory cytokines and TLR ligands. **A)** Expression of NOD2 mRNA in RASFs after incubation with various stimuli as measured by conventional PCR. One representative picture of 2 experiments is shown. **B)** Differential induction of NOD2 mRNA in RASFs (n=6) compared to OASFs (n=4) after incubation with various stimuli as measured by Real-time PCR. Differences between patient groups were calculated by Mann-Whitney U test, data are presented as mean \pm SEM. **C)** Western blot showing increased levels of NOD2 protein after stimulation of RASFs for 48h (n=3, one representative blot shown).

Figure 4. Levels of IL-6 and IL-8 are up-regulated after stimulation of RASFs with MDP. Protein levels of **A)** IL-6 and **B)** IL-8 in supernatants of RASFs (n=7) stimulated with

TNF, IL-1 or TLR ligands alone (stim), or in combination with MDP (stim + MDP). **C)** RASFs (n=6) were stimulated with PIC for 5h, washed and then stimulated with increasing concentrations of MDP for 24h. Levels of IL-6 and IL-8 in the supernatants were measured by ELISA. **D)** Scrambled or NOD2 siRNA transfected RASFs (n=6) were stimulated with PIC for 5h, washed and then stimulated with MDP for 24h. Levels of IL-6 and IL-8 in the supernatants were measured by ELISA. Significant differences were calculated by Wilcoxon signed rank test, all data are presented as mean \pm SEM.

Figure 5. MDP signals via p38 and NF- κ B. **A)** RASFs (n=2) were stimulated with PIC for 5h, washed and then stimulated with MDP for 15, 30, or 60min. Levels of phosphorylated and total p38, JNK and ERK were measured by Western blot and quantified by densitometric measurements. **B)** RASFs were stimulated with PIC for 5h, washed and then stimulated with MDP with or without addition of a pharmacological inhibitor of p38, ERK or JNK for 24h. Levels of IL-6 (n=5) and IL-8 (n=6) were measured by ELISA. **C)** Nuclear extracts from RASFs stimulated with PIC for 5h, washed and then stimulated with MDP for 30 and 60 min were used in an EMSA with a biotin- labeled NF- κ B probe and an unlabelled (cold) probe to exclude unspecific binding.

Figure 6. Stimulation with MDP induces the expression of MMP1, MMP3 and MMP13 in RASFs. **A)** RASFs (n=6) were stimulated with MDP and/or PIC and changes in mRNA expression of MMP1, MMP3, and MMP13 were measured by Real-time PCR. **B)** Total amount of MMP3 protein in the supernatants of unstimulated RASFs and RASFs stimulated with MDP and/or PIC (n=10) as measured by ELISA.. **C)** Levels of MMP1, MMP3 and MMP13 mRNA were significantly diminished after 5h pre-stimulation with PIC and 24h stimulation with MDP in NOD2 silenced RASFs when compared to control transfected

RASFs (n=7). Significant differences were calculated by Wilcoxon signed rank test, all data are presented as mean \pm SEM

Figure 1

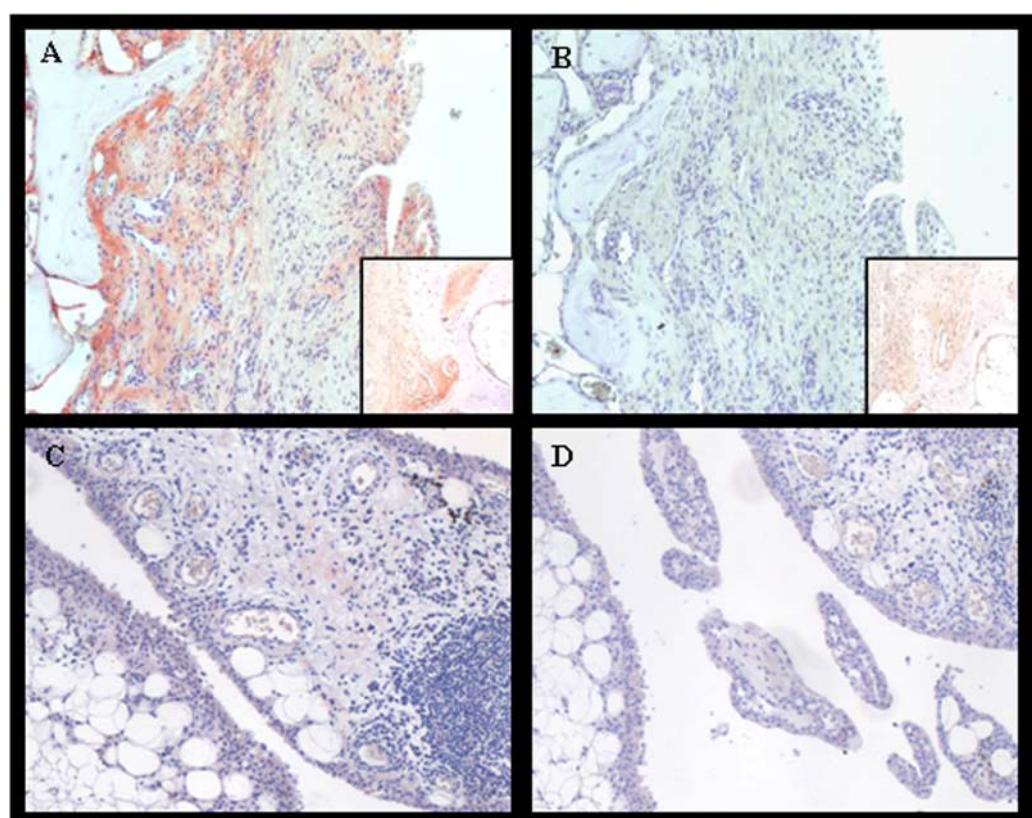


Figure 2

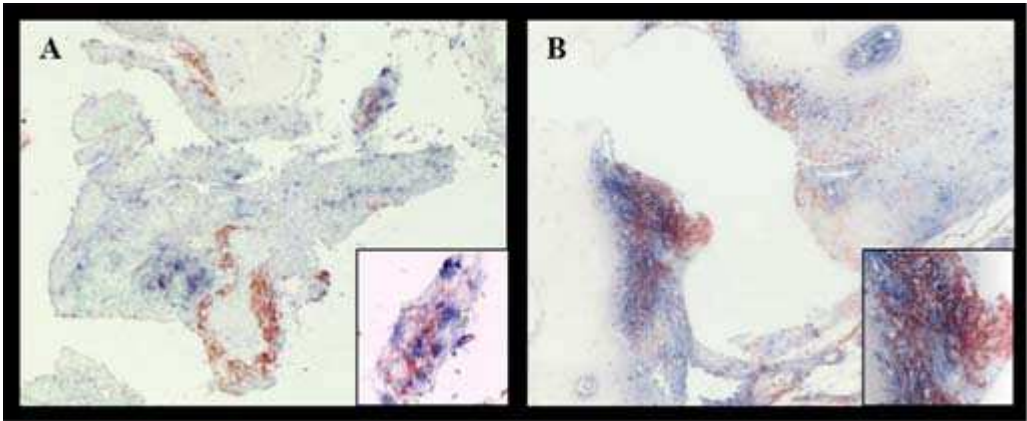


Figure 3

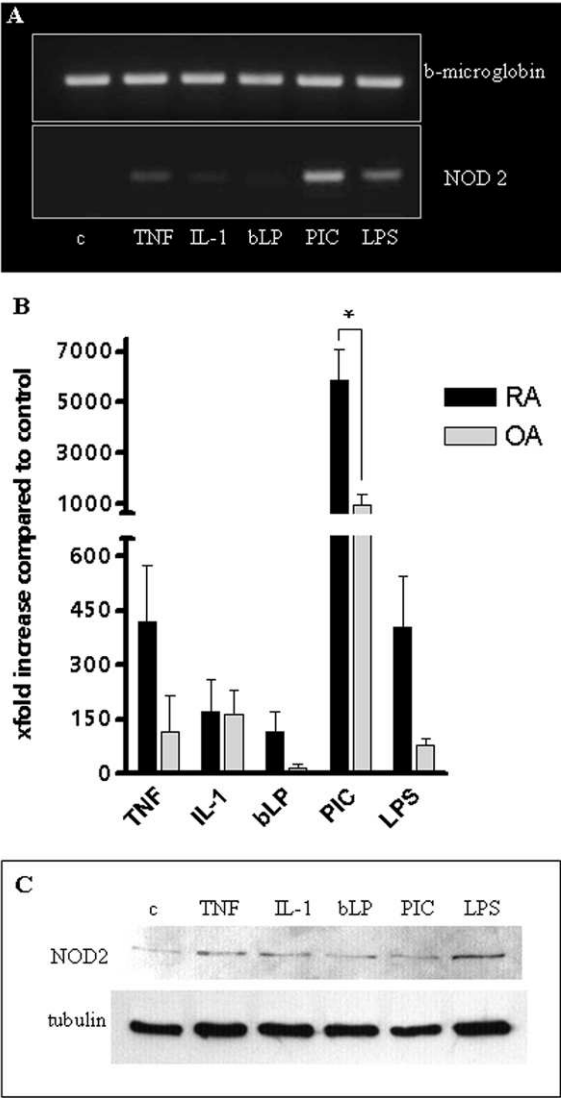


Figure 4

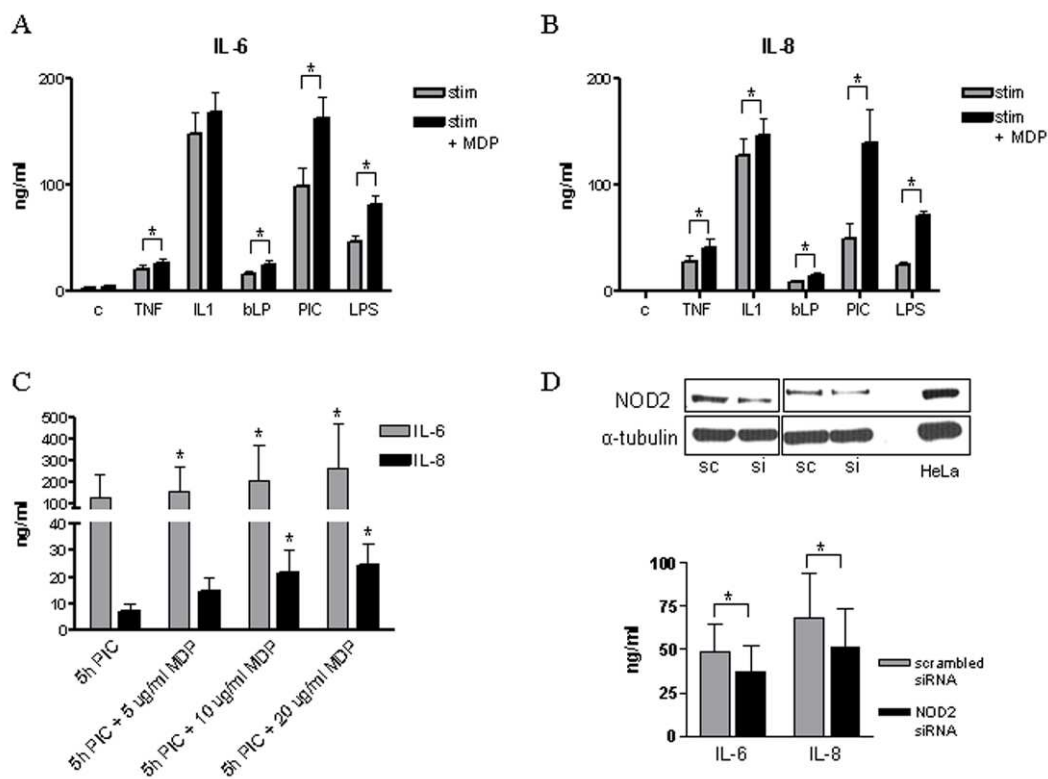


Figure 5

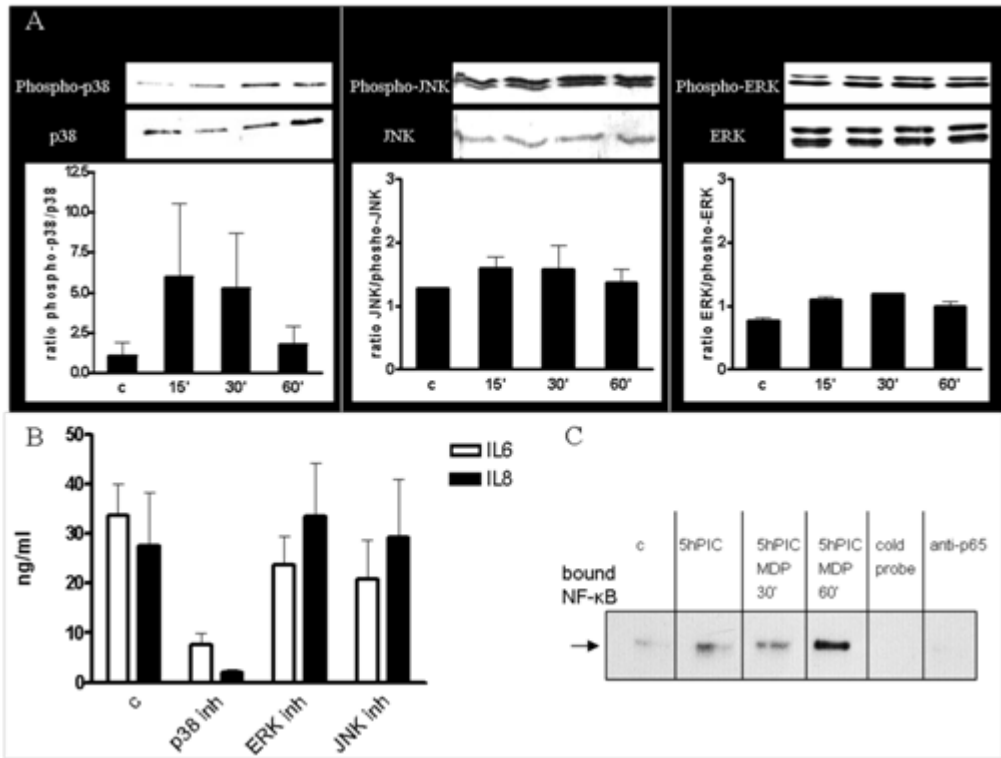


Figure 6

